

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification⁶ : A01N 43/16 // (A01N 43/16, 43:16, 25:24, 25:10)</p>	<p>A1</p>	<p>(11) International Publication Number: WO 99/40790 (43) International Publication Date: 19 August 1999 (19.08.99)</p>
<p>(21) International Application Number: PCT/US98/09027 (22) International Filing Date: 8 May 1998 (08.05.98) (30) Priority Data: PCT/US98/02725 12 February 1998 (12.02.98) WO (34) Countries for which the regional or international application was filed: GH et al. (71) Applicant (for all designated States except US): DCV, INC. [US/US]; 3521 Silverside Road, Wilmington, DE 19810 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): HEINSOHN, George, E. [US/US]; 6 Gilmore Lane, Elkton, MD 21921 (US). BJORNSON, August, S. [US/US]; 4616 Weldin Road, Wilmington, DE 19803 (US). (74) Agent: WINSLOW, Don, O.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report.</p>
<p>(54) Title: METHOD FOR TREATING COTYLEDONOUS PLANTS</p> <p>(57) Abstract</p> <p>Application of an aqueous solution containing a matrix polymer and oligomers of chitosan to the foliage of growing plants increases the yield of vegetables, tubers, cereal grains, fruits, and blossoms. Plants so treated are healthier, sturdier, more resistant to drought, and many varieties enjoy an extended period of production. The plants may be effectively and conveniently treated by spraying the foliage with a solution containing 0.005 % to 5.0 % by weight matrix polymer and chitosan oligomers in such amount that the weight ratio of chitosan oligomers to matrix polymer is 0.01 to 10.0.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

5

TITLE**METHOD FOR TREATING COTYLEDONOUS PLANTS**

10

FIELD OF INVENTION

The Invention is directed to a method for treating cotyledonous plants to improve the yield, health, and vigor of the plant by spraying on the leaves an aqueous solution of chitosan oligomers in a matrix polymer.

15

BACKGROUND OF THE INVENTION

It has been amply demonstrated that application of chitosan salts to the seeds of cereal crops results in dramatic changes in the biochemistry of the emergent plant. Included among these changes are (1) increased production of a class of compounds known as the phytoalexins which provide protection against localized microbial infection, and (2) increased production of callose and lignin, which provide structural strength and function as a barrier to the spread of infection. These changes occur as a result of activation of the gene encoding the enzyme *phenylalanine ammonia lyase* which is involved in the rate determining step of the phenylpropanoid metabolism pathway. Chitosan salts have been shown to induce synthesis of the terpenoid phytoalexins which are closely involved in the biosynthesis of growth hormones such as gibberelic acid and abscisic acid. Chitosan salts also induce activation of genes which produce chitinase and glucanase enzymes that are known to be fungal inhibitors and to play a role in pollen development and seed germination, as well as activation of genes which produce protease inhibitors that help protect the plant from insect attack. On a macroscopic scale, these changes translate into enhanced root

20

25

30

development, reduced lodging (plants falling over before harvest), enhanced yield, and greater resistance to certain plant diseases.

US 4,812,159 (Freepons) discloses treatment of soil in the seed planting zone with a solution of chitosan, application of a chitosan solution to plant seeds, treatment of soil in the seed planting zone with a mixture of solid chitosan and a solid acid, treatment of soil in the seed planting zone with a solid chitosan salt, and application of chitosan salts to the leaves of emerging plants.

US 4,964,894, which is a continuation-in-part of US 4,812,159, describes again the same techniques noted above and then discloses a procedure involving seed germination studies for identifying non-phytotoxic acids. Another technique using chitosan is described in US 5,554,445 (Kivekas, Struszczyk) which involves spraying seeds with a liquid dispersion of microcrystalline chitosan, followed by drying to form a polymer film around the seed.

US 4,886,541 (Hadwiger) discloses the application of a chitosan preparation to wheat seed in order to enhance yield, reduce lodging, and enhance root development. US 4,978,381 (Hadwiger) and US 5,104,437 (Hadwiger) describe the same techniques for seed treatment and provide additional examples to extend the method to other cereal crops such as rice, oats, barley, and rye.

Chitosan has been employed in agriculture for purposes other than its ability to enhance crop yields. For example, it has demonstrated ability as a bactericide against a variety of microorganisms and has been employed extensively for this purpose. In addition, the use of chitosan to inhibit bacteria-nucleated frost damage is disclosed in JP 07179843 A (assigned to Daiicche Seimo KK), and US 5,374,627 discloses the use of a chitosan hydrolysate of molecular weight (MW) 10,000- 50,000 prepared by high- temperature, acid-catalyzed degradation of chitosan as an effective agent for protecting plants against a number of plant diseases such as bacterial soft rot in vegetables, spring deadspot in turfgrass, and bacterial grain rot in rice.

US 5,726,123 discloses a method for treating plants by foliar application

of a solution containing both a chitosan salt and oligomers of chitosan that results in increased yield, better draught resistance, and improved overall vigor. The chitosan salt is prepared from chitosan of molecular weight greater than 50,000 amu (atomic mass units, daltons) and an acid that forms a water-soluble chitosan salt. The chitosan oligomers are defined as short fragments of chitosan chains having a degree of polymerization of 2-50, which corresponds to a molecular weight of approximately 320-10,000 amu. While application of the techniques disclosed in US 5,726,123 convey significant benefit to the user, there are certain drawbacks to employing the described technology. Chitosan is relatively expensive; it dissolves in dilute acids only with difficulty; and results in solutions with very high viscosity. Special techniques for preparing chitosan salt solutions are described in US 5,726,123, but even when these techniques are employed it is difficult to get flowable solutions of more than a few percent concentration. This limit on concentration poses significant operational problems for application in the field on a commercial scale. To apply the formulation to a crop, large quantities of dilute solutions containing chitosan salt and chitosan oligomer must be formulated, packaged, shipped to the grower, stored, transported to the field, transferred into spraying equipment, and applied to the crop at considerable economic penalty. Modern agricultural practice is ill suited for this type of operation because it generally employs equipment that is designed to work with highly concentrated agrichemicals that are typically 30-80 % active agent. Aside from the purely economic factors noted above, working with an agrichemical at low concentrations would necessitate changes in cultural practice that would make marketplace acceptance difficult.

SUMMARY OF THE INVENTION

This invention is directed to the application of a solution containing both chitosan oligomers and a water soluble polymer to the foliage of cotyledenous growing plants to enhance their yields. Plants so treated are healthier, more

drought resistant, and many varieties enjoy an extended production period. The treatment may be repeated throughout the growth cycle of the plant, especially during critical stages of plant growth such as flowering, seed formation, and ripening.

The invention is, therefore, directed primarily to a method for improving the yield, health, and vigor of growing cotyledonous plants comprising spraying onto the foliage between appearance of the first true leaves and completion of the harvest of the plant or fruit therefrom an aqueous solution containing dissolved therein 0.01 to 5.0% chitosan oligomers and a water soluble polymer. Optionally, the application is repeated before harvesting the plant or useful portion thereof.

DETAILED DESCRIPTION OF THE INVENTION

A. Introduction

U.S. 5,726,123 describes the beneficial effects achieved by applying a solution containing both low molecular weight (MW <10,000) chitosan oligomers and a high molecular weight (MW >50,000) salt of chitosan to a growing plant, preferably by foliar spraying. From the earlier descriptions of the dramatic alterations of plant biochemistry produced by exposure to a chitosan salt, it is evident that the chitosan salt effects changes in the plant by a systemic mechanism. This means that the active agent must penetrate the membrane defining the surface of the seed or plant. A chitosan salt applied to the foliage of a plant would, therefore, have to penetrate the epidermal layers in order to initiate any systemic processes. It is problematic how a very large molecule such as a chitosan salt of >50,000 amu would be able to effect epidermal penetration.

We now have evidence that most of the observed biological enhancement observed when solutions containing both a chitosan salt and chitosan oligomers are used is due to the low MW chitosan oligomers which are more easily able to permeate the epidermal layer, while the high MW chitosan salt

5 serves mainly as a matrix to bind the oligomers to the plant and to inhibit loss of water-soluble oligomers from the plant during rainfall or heavy dew. The chitosan salt also provides a minor source of chitosan oligomers derived by the natural degradation processes that cleave the chitosan chains into smaller and smaller fragments.

10 A direct consequence of this finding is that since the active agent (chitosan oligomers) is the minor constituent of the mixtures earlier disclosed, new formulations may be devised which contain less chitosan salt relative to chitosan oligomers than those described in U.S. 5,726,123, and hence a higher concentration of active agent at a given chitosan salt concentration. Since it is the high MW chitosan salt that is the principal determinant of solution viscosity, this means that solutions may be formulated with a higher
15 concentration of active ingredient at a lower viscosity, thereby overcoming the limitations of working with dilute solutions noted earlier.

20 A second direct consequence is that the chitosan salt may be replaced by other polymers (matrix polymers) that are able to keep the chitosan oligomers from being washed from the plant upon exposure to water, and which may possess other advantages over chitosan salts in particular applications. While chitosan salts offer the advantages of serving as a continuous source of small quantities of chitosan oligomers through degradative processes, and becoming more moisture resistant with the passage of time, they have the disadvantage
25 of forming aqueous solutions of very high viscosity, even at low concentration. Solutions of other polymers at equivalent concentration can have lower viscosity, thereby avoiding the disadvantages noted above.

30 Chitosan salts are particularly well suited to the purpose of protecting chitosan oligomers from rainfall and dew. As mentioned above, films formed

5 from chitosan salts of volatile acids become more water-insoluble as the acid
evaporated. The initial film of water-soluble chitosan salt, which is formed on
the leaf surface as the foliar spray dries, becomes progressively less water-
soluble and, therefore, more resistant to rainfall and dew with the passage of
time. As this insolubilization continues, the chitosan oligomers contained
within the chitosan salt film become progressively less susceptible to being
washed from the leaf by rainfall or dew. The elapsed time over which this
10 occurs depends on the volatility of the acid and on the drying conditions
encountered in the field. Since evaporation is a surface phenomena, the acid
must diffuse through the chitosan salt in order to reach the surface where it
can evaporate. Consequently, the thickness of the film is also an important
factor in the rate at which insolubilization takes place. Because these variables
cannot be rigidly controlled under field conditions, the timing of chitosan salt
15 insolubilization in field use cannot be precisely specified. Using the preferred
chitosan acetate salt and the spraying protocol defined in the examples under
normal field conditions, the loss of water solubility becomes evident in a few
hours and in most cases is essentially complete within a few days.

20 The ability to keep chitosan oligomers bound to the plant and protected
from moisture is not limited to salts of chitosan with volatile acids.
Evaporation is not the sole mechanism for loss of acid from a chitosan salt
film. Acid may also be removed from the chitosan salt film by biodegradation,
through degradation by ambient conditions such as by hydrolysis and/or
25 photolysis, or by deliberate treatment with basic materials such as ammonia
and calcium carbonate which may be incorporated in the spray or introduced
as a post treatment. Consequently, almost any acid which forms water soluble
salts with chitosan is suitable. A further description of suitable acids is
detailed below. While chitosan salts are indeed suitable for protecting chitosan
30 oligomers from dew and rainfall, there are associated disadvantages caused by

the high viscosity of chitosan as previously noted. With the knowlege that the chitosan oligomers are supplying the bulk of activity, it is now possible to prepare formulations with concentrations of active ingredient comparable to other agrichemical products (30%-80% wt.) without incurring the penalties associated with high viscosity. This can be done because the chitosan oligomers readily dissolve in water at high concentration, and the resulting solutions are not of inordinately high viscosity. A relatively small amount of chitosan salt is sufficient to bind the oligomers to the plant and protect them from moisture, thereby avoiding high viscosity.

It is a generally observed phenomena that high molecular weight polymeric materials are slow to dissolve even in solvents in which they can ultimately be dissolved in high concentration. A number of factors are involved in this apparent discrepancy. One of the more obvious factors is that the long polymer chains are intricately intertwined so that a molecule which has one of its ends at the surface and readily available for solvation, may have its opposite end deeply buried within the solvent-deprived interior defined by the bulk phase of the polymer. Dissolution cannot occur until solvent has diffused into the interior to solvate the protected part of the polymer chain. The languorous nature of the diffusion process is partly responsible for the slow rate of polymer dissolution. For purposes of the present invention, this phenomenon can be employed in useful fashion. Chitosan oligomers may be embedded in a wide variety of water soluble polymers other than chitosan salts and still be protected from rainfall and dew by the slow rate at which the polymer dissolves. Provided that the moisture exposure is of relatively limited extent (as is often the case with dew or light showers), the polymer, by its slowness in dissolving, will provide protection for the oligomers even though it would ultimately fail under more strenuous conditions such as prolonged periods of heavy rainfall.

B. Chitosan Oligomers

Techniques are well known in the literature for causing scission of chitosan chains using acid or enzyme catalysts. Depending on the starting material and reaction conditions chosen, the extent to which chain scission occurs can be controlled so that the resulting fragments are large enough to be considered still as chitosan (DP>250). At the other extreme, chain scission can be conducted under conditions so rigorous that the only products are the monomeric species D-glucosamine and N-acetyl-D-glucosamine (DP=1). For present purposes, the term "chitosan oligomers" is intended to refer to those homopolymers and copolymers of D-glucosamine and N-acetyl-D-glucosamine having a degree of polymerization (DP) of 2 to 50 corresponding to molecular weight of about 320 to 10,000 amu. Procedures for preparing such chitosan oligomers by acid catalysis have been described by Horowitz, Roseman, and Blumenthal (J. Amer. Chem. Soc., 1957, 79, 5046-49). Procedures for preparing such chitosan oligomers by enzymatic cleavage have been described by Li, Brzezinski, and Beaulieu (Plant Physiol. Biochem., 1995, 33 (5), 599-603). The chitosan oligomers used in the present study were prepared by a modification of the methods of Li, et al. Chitosan oligomers were prepared by adding chitosanase enzyme (from the actinomycete *Kitasatosporia* N174) to a solution of chitosan acetate at 37-55C. After 31 hr., the mixture was heated to 85C to denature the enzyme, and the product concentrated under vacuum at 35-40C and then lyophilized to dryness. Size exclusion chromatography on a Supelco-TSK Column G-Oligo-PW with a mobile phase containing 0.03 M acetic acid and 0.2 M sodium sulfate revealed that 41% of the resolved oligomers were of DP (degree of polymerization)<7 and 59% of DP 6-28.

C. Matrix Polymers

1. In General:

Suitable polymers for forming a matrix around the chitosan oligomers

must be environmentally innocuous, and also be water soluble in order to facilitate preparation of a solution to spray on a plant. These polymers must also be able to attach to the surface of the leaf, and should therefore be capable of forming hydrogen bonds with polar sites such as -OH on the leaf surface. Polymers containing -OH, -NH, or other polar functional groups such as carbonyl and amido groups are capable of such hydrogen bonding. A detailed review of structural features essential to hydrogen bonding is found in **Advanced Organic Chemistry** by J. March, McGraw-Hill, N.Y., N.Y., 1977, pp. 75-78, hereby incorporated by reference. In order to prevent the oligomers from being washed from the plant by moderate rainfall or dew, the polymer must dissolve slowly enough in water to provide the necessary protection under field conditions. Different chemical classes of water soluble polymers vary greatly in the rates at which they dissolve. But even within a given class of polymers, the rate of dissolution may vary widely because of differences in such characteristics as molecular weight and the nature of terminal groups at the end of the polymer chain. Therefore, suitable candidates for use as matrix polymers are those which (1) can be dissolved at a concentration of 0.5% wt. or higher in water, (2) are environmentally innocuous, (3) contain functional groups capable of forming hydrogen bonds, and (4) meet the following insolubility criteria:

Cast a film of the polymer 0.2 mm thick on a roughened plate by evaporation of solvent from an aqueous solution of the polymer. This may be accomplished by spotting approximately 2 mL of 4% polymer solution onto an area of 4 cm². Other combinations of solution concentration, sample size, and area may be used if desired so long as approximately 20mg polymer per cm² is delivered. For polymers of low solubility, multiple spotting may be required with partial evaporation of solvent between applications. Dry the film at to constant weight at 50-70C, and determine the weight of the film by

5 difference. Immerse the film in water at 22C for 0.5 hour without agitation, then remove the plate and again dry to constant weight. Determine the weight of polymer remaining on the glass by difference. If more than 40 % of the polymer in the original cast film remains, the candidate polymer has sufficient resistance to dissolution to be suitable as a matrix polymer for protection of chitosan oligomers.

10 Provided they meet the above criteria, suitable matrix polymers may be naturally occurring or synthetic, or mixtures thereof. Among the classes of natural polymers that will include suitable members are soluble polysaccharides, alginates, polyhydroxyacids, polyaminoacids, polyaminosaccharides and soluble chitosan salts. Other suitable naturally occurring polymers will be obvious to one skilled in the art. Among the synthetic polymers that will include suitable members are polyethers, 15 polyacetals, polyesters, and polyamides, and poly(alkoxy cellulose) compounds, such as carboxy methyl cellulose. Other suitable synthetic polymers will be obvious to one skilled in the art.

20 2. Chitosan as a Matrix Polymer

25 Though chitosan is a naturally occurring polymer found in many fungi, it is neither abundant nor readily isolated from natural sources in high purity. As a matter of convenience, chitosan is more readily obtained from chitin which, after cellulose, is the most abundant natural polymer. Chitin is readily isolated from shellfish or insect exoskeletons, and is also found in mollusks and fungi. A convenient method of obtaining chitosan from the chitin found in shellfish waste is described in U.S. 3,862,122 (Peniston). Although there is a continuum of compositions possible between the pure homopolymers, poly-N-acetyl-D-glucosamine and pure poly-D-glucosamine residues. For present purposes, the term "chitosan" is intended to apply to copolymers of D- 30 glucosamine and N-acetyl-D-glucosamine containing 0-35% N-Acetyl-D-

glucosamine residues and having a molecular weight greater than 50,000 amu and corresponding to polymer chains in which about 250 or more monomer units are joined together (degree of polymerization or DP = 250). Since the D-glucosamine residues of chitosan bear a basic amino function, they readily form salts with acids. Suitable acids for making the chitosan salts for use in the invention are those which form water-soluble salts with chitosan. It is not necessary that the acid itself be water-soluble; however, such water-soluble acids are preferred because of ease in handling them. Inorganic acids which form water-soluble chitosan salts include the halogen acids and nitric acid but exclude sulfuric and phosphoric acids because they do not form water-soluble salts with chitosan. Organic acids are preferred and include the following: lactic acid, glycolic acid, glutamic acid, and acetic acid. Acetic acid is most preferred because it is inexpensive, environmentally innocuous, and sufficiently volatile so that insolubilization of the corresponding chitosan salt is readily accomplished.

Commercially available chitosan is typically prepared from shellfish and has a molecular weight measured in the hundreds of thousands, corresponding to polymer chains in which several thousand monomer units are linked together in β -1,4 fashion. Chitosan obtained from fungal sources is typically of somewhat lower molecular weight and may contain fractions with molecular weight as low as 50,000 amu (atomic mass units, daltons). The chitosan used in this study was obtained from shrimp or crab shell, and contained about 75-82% D-glucosamine residues which is typical of

commercially produced chitosan. It was readily soluble in dilute aqueous solutions of mineral and carboxylic acids.

5 **D. Additives**

As used herein, the term "additives" refers to materials which may be optionally used to augment the effectiveness of the invention, but do not themselves have bio-activity. These include such materials as surfactants, wetting agents, defoaming agents, extenders, penetrants, activators, spreading agents, diluents, odorants, brightening agents and the like. It is particularly preferred to use a small amount of wetting agent in the compositions in order to obtain even distribution and wetting of the hydrophobic surface of the plant leaves. Such agents are usually used in concentrations of 0.01 – 0.1% by weight.

15

E. Coadjuvants

As used herein, the term "coadjuvant" refers to materials that have a bio-activity that may be the same or different than the bio-activity of the chitosan salts. Such materials include fertilizers, fungicides, insect repellants, plant hormones, trace nutrients, pesticides, herbicides, and mixtures thereof. Both liquid and solid coadjuvants can be used in conjunction with water-soluble matrix polymers and oligomers, so long as the resultant aqueous compositions are sprayable.

25

E. Oligomer/Matrix Polymer Solutions

A mixture of chitosan oligomers and a suitable matrix polymer may be applied to the foliage of growing plants by spraying with an aqueous solution containing appropriate amounts of these materials, or by other techniques which will be readily apparent to one skilled in the art. The preferred technique is by solution spraying which eliminates the problems associated with settling of dispersions, or the dust inhalation hazard associated with applying solids to the

30

plant. It is preferred to use spray solutions having a matrix polymer concentration of 0.005 % to 5.0%, and most preferred to use solutions with concentration of 0.01% to 0.5 % . Solutions in this concentration range have viscosities compatible with the use of commercial spraying equipment . It is preferred to use solutions in which the weight ratio of chitosan oligomers to matrix polymer is in the range 0.01 to 10.0 and preferably in the range 0.05 to 5.0. Solutions in this range of concentration require an application rate of about 5-150 gallons per application per acre to achieve correct dosage which is compatible with the normal operation of commercial spraying equipment. As a matter of convenience, it is generally desirable to prepare a concentrate containing chitosan oligomer and matrix polymer which may be diluted with water at the site of use prior to application to the plant. For typical field operations, it is convenient to dilute the concentrate with 100-500 volumes of water, so the concentrate should be prepared so as to contain the correct amount of chitosan oligomer and matrix polymer after such dilution.

For most matrix polymer/chitosan oligomer combinations, preparation of the concentrated solution is straightforward. The desired amount of matrix polymer is dissolved in the appropriate amount of water, cooled to ambient temperature if heating has been employed to aid in dissolving the matrix polymer, and the appropriate amount of chitosan oligomers added with mild agitation. When a chitosan salt is chosen as the matrix polymer, a more specialized technique is employed since the chitosan must be converted into a salt during the dissolution process. A preferred method when a chitosan salt is chosen as the matrix polymer is to form an aqueous dispersion of chitosan at a temperature of 45-85 C and preferably at 55-75C and to then add the acid. It is preferred that the reaction temperature be at least 45C in order to have a rapid rate of reaction without the necessity of using a large excess of acid. On the other hand, it is preferred that the reaction temperature not exceed 85 C in order to avoid

discoloration and to assure stability of the water-soluble salt. Chitosan oligomers, additives, and coadjuvants can be added to the reaction solution at any stage, but it is preferred that they be added after the reaction is complete and the solution has
5 cooled in order to minimize any secondary reactions.

It is not necessary to evaporate the applied solution. In fact, it is preferred not to do so for the reason that adsorption of the chitosan oligomers into the plant takes place rapidly from the liquid state. However, evaporation of the solution to
10 apparent dryness will ordinarily take place due to normal atmospheric conditions. The amount of residual moisture remaining in the matrix polymer will depend on the chemical nature of the matrix polymer as well as the temperature and relative humidity. In all but the most extreme field conditions, some moisture will be
15 retained by the matrix polymer and thereby provide a mechanism for conveying the chitosan oligomers to the interior of the plant.

G. Treatable Plants

A wide variety of cotyledonous plants can be advantageously treated by the method of the invention, so long as the treatment is carried out in the manner
20 described above. Such plants include members of the genera *Allium*, *Appium*, *Asparagus*, *Beta*, *Brassica*, *Capsicum*, *Citrullis*, *Cucurbita*, *Daucus*, *Frageria*, *Lactuca*, *Lycopersicum*, *Phaseolus*, *Solanum*, *Spinachia* and *Zea*. Among the members of these genera of vegetables are asparagus, beans, beets, broccoli, carrots, celery, corn, egg plant, lettuce, melons, onions, pea, peppers, potatoes,
25 spinach, squash, strawberries and tomatoes.

H. Safety

A further advantage of the invention is that the chitosan oligomers are non-toxic. By choosing a matrix polymer that is of similar low toxicity (e.g. a
30 polysaccharide), the compositions of the invention will be non toxic to birds,

mammals, or humans. Moreover, the low toxicity level and easy biodegradability of the compositions act to prevent detrimental effects on the beneficial constituents of fertile soil layers. Accordingly, EPA has established an exemption
5 from the requirement of a tolerance for residues of poly-D-glucosamine, when they are used in the production of raw agricultural commodities.

EXAMPLES

10 **Test Procedure**

A chitosan acetate solution was prepared by vigorously stirring the appropriate amount of water at a temperature of 60 C and adding small flakes of chitosan containing 80 % D-Glucosamine residues at such a rate that the chitosan became wetted and dispersed throughout the liquid phase. Glacial acetic acid
15 was then added in the ratio of 0.36 lb. of acetic acid per pound of chitosan. This represents a ratio of 1.03 equivalents of acetic acid per mole of amino function in the chitosan. The mixture was stirred at 60 C until substantially all the chitosan had dissolved, and the resulting solution then filtered through coarse cheese cloth to remove any adventitious particles. The quantities of water and chitosan were
20 chosen so that the chitosan concentration (exclusive of the acetate anion) was 5.0 %. A portion of the resulting solution was diluted to a chitosan concentration of 2.5 % and used in Examples I and II to illustrate the effect of chitosan salt only on the test plants. Solid chitosan oligomers were added to a second portion of the chitosan acetate solution equivalent to 5 % of the weight of chitosan (exclusive of
25 acetate anion) and the mixture briefly agitated to ensure dissolution of the oligomers. This solution was then further diluted with water to a total glucosamine (chitosan salt plus chitosan oligomers) concentration of 2.5 % and used in Examples I and II to illustrate the effect of chitosan oligomers dispersed in a chitosan acetate matrix at a 0.05 ratio of chitosan oligomers to chitosan
30 acetate.

Treatment was commenced as soon as the plant had produced its first set of true leaves. The true leaves are those which follow the emergent leaves (cotyledons) and which resemble the leaves of the mature plant in shape. The treatment protocol was to spray the foliage of the plant until the top of the leaf surfaces were thoroughly wetted and solution began to drip from the leaf tips, and to repeat the treatment approximately every 2 weeks, as weather conditions allowed. As the plants increased in size, it was obviously necessary to use more chitosan spray solution to wet the leaves. It was estimated that the treatment rate was 10-20 gallons/acre for the initial treatment and 40-60 gallons/acre at the end of treatment.

The following examples are intended to demonstrate that most of the yield increase noted when a mixture of a chitosan salt and chitosan oligomers is applied to a plant is due to the presence of chitosan oligomers, while the chitosan salt makes only a small direct contribution to the beneficial result.

Example I

This test was designed to show the effect of plant treatment with a chitosan salt alone, compared to an untreated control and to treatment with a mixture of chitosan salt and chitosan oligomers.

The solution containing 2.5 % chitosan acetate described hereinabove was diluted 17:1 with water. Plants treated with this solution were designated group A. The solution containing 2.5 % of chitosan acetate plus chitosan oligomers described in paragraph I was diluted 17:1 with water. Plants treated with this solution were designated group B. Untreated plants were designated as group C.

Bell peppers ("Yolo Wonder") were treated with the appropriate solution when the first true leaves emerged 18 days after seeding. They were transplanted into the field in random block fashion such that each treatment protocol was replicated eight times with 10 plants in each replicate, so that each test was

performed 80 times. Spray treatments with the appropriate solution were applied every two weeks until harvest. The crop was harvested by hand over a period of several weeks, and total yield for each treatment protocol arrived at by
 5 summation.

TREATMENT	TOTAL YIELD, Grams	INCREASE vs UNTREATED
A (Chitosan Acetate)	22478	1.4%
B (Chitosan Acetate + Chitosan Oligomers)	26745	20.6%
C (Untreated)	22170	_____

From the above data it may be concluded that treatment of the plants with chitosan acetate alone produced only a slight increase in yield. Inclusion of chitosan oligomers in the chitosan acetate matrix results in a dramatic yield
 10 increase that cannot be attributed except in minor part to the chitosan acetate.

Example II

Cucumbers ("Milligon") were raised in a commercial greenhouse and treated every two weeks with the solutions described in Example I containing
 15 either (A) chitosan acetate alone or (B) containing both chitosan acetate and chitosan oligomers. Each test group contained 400 plants. The group treated with chitosan acetate alone afforded an average of 621 fruits per 100 plants with 11.6% classified by the grower as "small". These results were not significantly different from the untreated control. The group treated with a mixture of chitosan

acetate and chitosan oligomers afforded an average of 777 fruits per 100 plants with 3.9 % classified by the grower as "small". This represents an increase of 25.1% in the number of fruits for the case where chitosan oligomers are
5 incorporated into a chitosan acetate matrix compared to the case that where only the chitosan acetate matrix polymer was applied. An additional benefit is that fewer undersized fruits were harvested in the case using chitosan oligomers.

CLAIMS

What is claimed is:

- 5 1. A method for improving the yield of growing cotyledenous plants having their first true leaves comprising (1) applying to the exposed surface of the leaves an aqueous liquid solution having dissolved therein 0.005-5.0% by weight of a water soluble matrix polymer and having also dissolved therein chitosan oligomers in such amount that the weight ratio of chitosan oligomers to matrix
- 10 polymer is 0.01 to 10.0, and optionally (2) repeating step (1) at least one time before harvesting the plant.
2. The method of claim 1 in which the matrix polymer is a naturally occurring water soluble polymer.
- 15 3. The method of claim 1 in which the matrix polymer is selected from the group consisting of polysaccharides, polyaminosaccharides, polyaminoacids, and alginates.
- 20 4. The method of claim 1 in which the matrix polymer is a synthetic water soluble polymer.
5. The method of claim 1 in which the matrix polymer is selected from the group consisting of polyhydroxy alkanes, polyhydroxy ethers, and carboxyalkyl
- 25 polysaccharides.
6. The method of claim 1 in which the matrix polymer is a chitosan salt prepared by reaction of chitosan with an acid.

7. A composition for treating cotyledonous plants having their first true leaves comprising an aqueous solution containing 0.005% to 5.0% by weight of a water soluble matrix polymer and having also dissolved therein chitosan oligomers in such amount that the weight ratio of chitosan oligomers to matrix polymer is 0.01 to 10.0.

8. The composition of claim 7 in which the matrix polymer is a naturally occurring water soluble polymer.

9. The composition of claim 7 in which the matrix polymer is selected from the group consisting of polysaccharides, polyaminosaccharides, polyaminoacids, and alginates.

10. The composition of claim 7 in which the matrix polymer is a chitosan salt prepared by reaction of chitosan with an acid.

11. The composition of claim 7 in which the matrix polymer is a synthetic water soluble polymer.

12. The composition of claim 7 in which the matrix polymer is selected from the group consisting of polyhydroxy alkanes, polyhydroxy ethers, and carboxyalkyl polysaccharides.

13. A growing cotyledonous plant, the first true leaves of which bear a layer of an aqueous liquid solution containing 0.005% to 5.0% by weight of a water soluble matrix polymer and having also dissolved therein chitosan oligomers in such amount that the weight ratio of chitosan oligomers to matrix polymer is 0.01 to 10.0.

- 14 The cotyledonous plant of claim 13 in which the matrix polymer is a naturally occurring water soluble polymer.
- 5
15. The cotyledonous plant of claim 13 in which the matrix polymer is selected from the group consisting of polysaccharides, polyaminosaccharides, polyaminoacids, and alginates.
- 10 16. The cotyledonous plant of claim 13 in which the matrix polymer is a chitosan salt prepared by reaction of chitosan with an acid.
17. The method of claim 13 in which the matrix polymer is a synthetic water soluble polymer.
- 15
18. The method of claim 13 in which the matrix polymer is selected from the group consisting of polyhydroxy alkanes, polyhydroxy ethers, and carboxyalkyl polysaccharides.
- 20 19. The method of claim 1 in which at least a portion of the plant is edible by humans.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/09027

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01N43/16 //(A01N43/16, 43:16, 25:24, 25:10)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 97, no. 10, 31 October 1997 & JP 09 143013 A (YAIZU SUISAN), 3 June 1997 see abstract	1-19
X	--- DATABASE WPI Section Ch, Week 8812 Derwent Publications Ltd., London, GB; Class C03, AN 88-080717 XP002077208 & JP 63 033310 A (IHARA CHEM IND CO LTD) , 13 February 1988 see abstract --- -/-	1-19

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

11 September 1998

Date of mailing of the international search report

22/09/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Lamers, W

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern. Patent Application No

PCT/US 98/09027

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 9141 Derwent Publications Ltd., London, GB; Class C03, AN 91-299396 XP002077209 & JP 03 198702 A (LION CORP) , 29 August 1991 see abstract ---	7-9
Y	EP 0 365 279 A (ALLIED COLLOIDS LTD) 25 April 1990 see page 2, line 13 - line 15 see page 2, line 38 - line 44 see page 3, line 47 - line 49 ---	1-19
Y	DATABASE WPI Section Ch, Week 8842 Derwent Publications Ltd., London, GB; Class C03, AN 88-296419 XP002077210 & JP 63 216804 A (MEIJI SEIKA KAISHA) , 9 September 1988 see abstract ---	1-19
Y	PATENT ABSTRACTS OF JAPAN vol. 96, no. 5, 31 May 1996 & JP 08 003011 A (TADAMORO INOUE), 9 January 1996 see abstract ---	1-19
Y	DATABASE WPI Section Ch, Week 8903 Derwent Publications Ltd., London, GB; Class C03, AN 89-020572 XP002077211 & JP 63 297305 A (KATAKURA CHIKKARIN CO LTD), 5 December 1988 see abstract ---	1-19
P,X	US 5 726 123 A (BJORNSEN AUGUST S ET AL) 10 March 1998 see the whole document ---	1-19
A	DATABASE WPI Section Ch, Derwent Publications Ltd., London, GB; Class A97, AN 73-05114U XP002077212 & JP 48 001878 B (SAKAMOTO M) see abstract --- -/--	1-19

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/09027

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *

Citation of document, with indication, where appropriate, of the relevant passages

Relevant to claim No.

A WO 89 07395 A (BENTECH LAB INC)
24 August 1989
see page 5, line 7 - line 24
see page 8, line 15 - line 33
see page 43; example XXXIV

1-19

A WO 92 08741 A (CENTRE NAT RECH SCIENT)
29 May 1992
see the whole document

1-19

A DATABASE WPI
Section Ch, Week 9540
Derwent Publications Ltd., London, GB;
Class A97, AN 95-307044
XP002077213
& JP 07 203771 A (NISHIKAWA GOMU KOGYO KK)
, 8 August 1995
see abstract

1-19

A DATABASE WPI
Section Ch, Week 9316
Derwent Publications Ltd., London, GB;
Class C03, AN 93-129167
XP002077214
& JP 05 065368 A (BIHOKU FUNKA KOGYO KK)
, 19 March 1993
see abstract

1-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. Appl. Application No

PCT/US 98/09027

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0365279	A	25-04-1990	AU 632767 B	14-01-1993
			AU 4351889 A	26-04-1990
			CA 2000972 A	18-04-1990
			DK 515389 A	19-04-1990
US 5726123	A	10-03-1998	WO 9834464 A	13-08-1998
WO 8907395	A	24-08-1989	AU 3192689 A	06-09-1989
			GR 89100093 A	31-03-1994
WO 9208741	A	29-05-1992	FR 2669340 A	22-05-1992

Form PCT/ISA/210 (patent family annex) (July 1992)

THIS PAGE BLANK (USPTO)